Effect of Pseudobactin 358 Production by *Pseudomonas putida* WCS358 on Suppression of Fusarium Wilt of Carnations by Nonpathogenic *Fusarium oxysporum* Fo47

PHILIPPE LEMANCEAU,1* PETER A. H. M. BAKKER,1 WILLEM JAN DE KOGEL,1 CLAUDE LABOUVETTE,2 AND BOB SCHIPPERS1

University of Utrecht, Department of Plant Ecology and Evolutionary Biology, Section of Plant Pathology, 3508 TB Utrecht, The Netherlands,1 and Institut National de la Recherche Agronomique, Laboratoire de Recherches sur la Flore Pathogène et la Faune du Sol, 21034 Dijon Cedex, France2

Received 7 April 1992/Accepted 22 June 1992

Nonpathogenic *Fusarium oxysporum* Fo47b10 combined with *Pseudomonas putida* WCS358 efficiently suppressed fusarium wilt of carnations grown in soilless culture. This suppression was significantly higher than that obtained by inoculation of either antagonistic microorganism alone. The increased suppression obtained by Fo47b10 combined with WCS358 only occurred when Fo47b10 was introduced at a density high enough (at least 10 times higher than that of the pathogen) to be efficient on its own. *P. putida* WCS358 had no effect on disease severity when inoculated on its own but significantly improved the control achieved with nonpathogenic *F. oxysporum* Fo47b10. In contrast, a siderophore-negative mutant of WCS358 had no effect on disease severity even in the presence of Fo47b10. Since the densities of both bacterial strains at the root level were similar, the difference between the wild-type WCS358 and the siderophore-negative mutant with regard to the control of fusarium wilt was related to the production of pseudobactin 358. The production of pseudobactin 358 appeared to be responsible for the increased suppression by Fo47b10 combined with WCS358 relative to that with Fo47b10 alone.

---

Fusarium wilt diseases are responsible for important yield losses on numerous crops. Inputs of agrochemicals used for crop protection are adversely affecting the quality of the food products and of the environment, thus making the development of alternative ways to control disease a high priority.

Some soils are known for their natural suppressiveness to fusarium wilts. Two of these soils, one in France (1, 24) and one in California (31, 34), have been described extensively. The soil microflora is responsible for the natural suppressiveness of these soils (24, 31). Two different microbial populations, nonpathogenic *Fusarium* spp. (30) and fluorescent *Pseudomonas* spp. (32), were demonstrated to be involved in soil suppressiveness. These antagonistic microorganisms, when introduced in conducive soil or growing substrate, significantly reduce fusarium wilt severity (2, 16, 29, 32, 37). However, the suppression with nonpathogenic *Fusarium* spp. or fluorescent *Pseudomonas* spp. never reaches that observed with the natural suppressive soils. Furthermore, biological control achieved with only one antagonistic microorganism is often associated with inconsistent positive results (33, 41). Since the natural suppressiveness of soil is not caused by only one microbial population (18, 33), several workers tried to control fusarium diseases with a combination of nonpathogenic *Fusarium oxysporum* and a fluorescent *Pseudomonas* sp. isolated from suppressive soils. Indeed, the biological control achieved with this combination was more efficient and consistent than that recorded after inoculation of the *Pseudomonas* sp. or nonpathogenic *F. oxysporum* alone (18, 19, 28).

To our knowledge, the mechanisms responsible for the beneficial effect of coinoculation of nonpathogenic *F. oxysporum* and fluorescent *Pseudomonas* spp. on disease suppression have never been studied. Numerous papers have demonstrated the involvement of pseudobactin production by fluorescent pseudomonads in biological control (7, 16, 20, 22, 26, 41). The aim of the present study was to assess the possible involvement of pseudobactin production by fluorescent *Pseudomonas* spp. in the efficient control achieved by a nonpathogenic *F. oxysporum* combined with a fluorescent *Pseudomonas* sp.

**MATERIALS AND METHODS**

Microorganisms and culture conditions. Nonpathogenic *F. oxysporum* Fo47b10 is a mutant of Fo47 that is resistant to benomyl (5 mg liter⁻¹) (13). The benomyl resistance is used as a specific marker to allow recovery of the introduced nonpathogenic *F. oxysporum* strain. The wild-type Fo47 was previously isolated from the Chaetangurard (France) soil that suppresses fusarium wilt; the efficacy of Fo47 in reducing the severity of fusarium wilt has been demonstrated several times (2, 8, 18, 19). Fo47 and Fo47b10 are equally effective in the control of fusarium wilt (13). WCS816 is a virulent strain of *F. oxysporum* f.sp. *dianthi* race 2. *F. oxysporum* Fo47b10 and WCS816 are single-spore isolates; they are cryopreserved by freezing conidial suspensions at −80°C in 50% glycerol. *F. oxysporum* strains were grown in malt extract liquid medium (10 g liter⁻¹) at 25°C. Because a higher amount of inoculum was required for Fo47b10 than for WCS816, Fo47b10 cultures were grown in a fermentor and WCS816 cultures were grown in erlenmeyer flasks on a reciprocal shaker. After 7 days of growth, cultures were
filtered through glass wool to remove mycelial mats. Microconidia left in the filtrate were pelleted by centrifugation (5,000 × g, 10 min) and rinsed three times with tap water. The conidial densities of suspensions of strain WCS816 were determined by direct observation on a hemocytometer. These microconidial suspensions of WCS816 were used to infest carnation plants (Dianthus caryophyllus L.). The strain Fo47b10 microconidial suspension was kept in talcum powder as described by Locke and Colhoun (21) and modified by Tello-Marquina et al. (35). The density of the Fo47b10 inoculum was assessed by dilution plating on malt extract-agar plates.

*Pseudomonas putida* WCS358 was isolated from the rhizosphere of potato plants (14). Its ability to promote growth of potato plant has been demonstrated (5, 6, 14). Strain JM218 is a siderophore-negative Tn5 transposon mutant of WCS358 (25). Cultures of bacterial cells, grown in Luria broth medium (27) either supplemented with kanamycin (200 mg liter⁻¹) for strain JM218 or not supplemented for strain WCS816, were stored at −80°C in 50% glycerol. Bacterial inoculants were produced on King’s medium B agar plates (15) at 25°C for 48 h. Bacteria were scraped from the medium and suspended in tap water, pelleted by centrifugation (5,000 × g, 10 min), and washed three times with tap water. The bacterial densities of the suspensions were measured by direct observation on a hemocytometer and adjusted by dilution.

**Treatment and plant growth conditions.** Carnation cuttings of the cultivar Lena (susceptible to *F. oxysporum* f.sp. *dianthi* race 2) rooted on rockwool granules were obtained from Van Staaveren B. V., Aalsmeer, the Netherlands. Plants were placed in Grodan rockwool cubes (365 cm³). Rockwool, a growing medium used for soilless culture, is made by melting rock (most commonly a form of basalt) at a temperature of 1,600°C and spinning it into fibers (39). Plants were grown in a glasshouse at 22°C with a photoperiod of 16 h and watered three times a week with a nutrient solution (11a) modified for iron (10 μM FeEDDHA). The volume of nutrient solution used was related to the evaporation and to the growth stage of the plants. The electrical conductivity and pH of the solution in the rockwool cubes were determined regularly and adjusted, if necessary, to 2.5 Ms cm⁻¹ and 6.5, respectively.

Cubes were first watered with 300 ml of nutrient solution containing 3.3 × 10⁴, 3.3 × 10⁵, or 3.3 × 10⁶ CFU of Fo47b10 ml⁻¹. The final density of Fo47b10 was 0, 2.7 × 10⁴, 2.7 × 10⁵, or 2.7 × 10⁶ CFU per ml of rockwool. One week later, plants were infested with pathogenic *F. oxysporum* (WCS816) and treated with bacterial strain WCS358 or JM218 by adding 20 ml of microbial suspension (5 × 10⁹ conidia of WCS816 ml⁻¹, 2.5 × 10⁶ bacteria ml⁻¹) to each cube. Control plants were infested with pathogenic *F. oxysporum* WCS816 and left untreated. The final density of WCS816 was 2.7 × 10⁶ CFU per ml of rockwool. The inoculum ratio of nonpathogenic *F. oxysporum* (Fo47b10) to pathogenic *F. oxysporum* (WCS816) in the rockwool cubes was 0:1 (infested control), 1:1, 10:1, or 100:1.

**Disease development.** The number of diseased plants was scored every week. A plant was considered to be diseased when the first local symptoms (chlorotic leaves, crook-neck shoots) had developed (3). Experiments were stopped when the disease severity of the infested control stopped increasing (9 to 10 weeks after infestation of plants with WCS816).

**Enumerations of antagonistic organisms.** The density of Fo47b10 in rockwool was determined 3 weeks after inoculation. A given volume of rockwool (43 cm³) was taken, always at the same location, from cubes and submitted to microbial analysis. Each sample was suspended in sterile water by mixing with a Waring blender for 30 s at high speed. A series of 10-fold dilutions was prepared from that suspension. An aliquot (1 ml) of each appropriate dilution was incorporated in malt extract-agar (15 g liter⁻¹) supplemented with citric acid (250 mg liter⁻¹) and benomyl (5 mg liter⁻¹) (9). For each dilution, 10 plates were poured. Colony numbers were recorded after 96 h of incubation at 25°C.

The densities of bacterial strains WCS358 and JM218 on the roots of carnations were determined 6 weeks after the bacterial treatment. Random samples of root pieces (0.3 g) were vigorously shaken in glass tubes containing 5 ml of 0.1 M sterile MgSO₄ - 7H₂O and 2.5 g of glass beads (0.17-mm diameter) for 1 min. An aliquot (0.2 ml) of each suspension was put in each of four different wells of a macroplate (22.6-mm well diameter; Costar) and incorporated homogeneously in King’s B-agar medium (1 ml per well) supplemented with 100 mg of cycloheximide liter⁻¹, 40 mg of ampicillin liter⁻¹, and 13 mg of chloramphenicol liter⁻¹ (14). After incubation (36 h at 27°C), the macrocolonies of WCS358 and JM218 were stained with fluorescein isothiocyanate-conjugated antibody as described by Van Vuurde and Roozen (38) and modified by Leeman et al. (17). A polyclonal antiserum was raised against whole cells of WCS358 at the Research Institute of Plant Protection (Wageningen, the Netherlands) by the method of Vrugink and Maas-Geesteranus (40). The absence of cross-reaction with indigenous pseudomonads demonstrated by Bakker (4) was checked with controls (no bacterial treatment). The reaction of the antiserum with the Tn5 mutant JM218 was also demonstrated. Transposon Tn5 encodes kanamycin and streptomycin resistance in bacterial strain JM218 (25). The retention of Tn5 in strain JM218 during the experiments was ascertained by comparing bacterial densities of this strain in root suspensions, estimated by the serological procedure described above, with bacterial densities estimated by plate counts on King’s B medium supplemented with cycloheximide (100 mg liter⁻¹) and kanamycin (200 mg liter⁻¹); at the same time, the absence of fluorescence of the JM218 colony grown on supplemented King’s B medium was confirmed.

**Experimental design and statistical analysis.** Microbial enumerations were performed on six replicates of two plants per treatment. Since populations of bacteria and fungi approximate a log normal distribution (9, 23), values were logarithmically transformed before analysis.

The experimental design for scoring disease severity was a randomized complete block with six replications of six plants per treatment. The percentages of diseased plants were submitted to angular transformation before statistical analysis.

Transformed values of microbial enumeration and of disease severity were analyzed by analysis of variance and then Fisher’s least significant difference test. Experiments were repeated at least twice.

**RESULTS**

**Effect of nonpathogenic *F. oxysporum* Fo47b10 on fusarium wilt severity.** Nonpathogenic *F. oxysporum* Fo47b10 reduced the severity of fusarium wilt (Fig. 1) when the density of nonpathogenic *F. oxysporum* Fo47b10 was at least 10 times higher than the density of pathogenic *F. oxysporum* WCS816. There was no improvement of the control achieved by nonpathogenic *F. oxysporum* Fo47b10 when the inocu-
lum ratio of nonpathogenic *F. oxysporum* to pathogenic *F. oxysporum* was increased from 10:1 to 100:1.

**Role of pseudobactin 358 production in suppression of fusarium wilt.** To determine the involvement of pseudobactin 358 production in suppression of fusarium wilt, bacterial strain WCS358 and its siderophore-negative (Sid−) mutant JM218 were compared for their ability to control fusarium wilt in the absence or presence of increasing densities of nonpathogenic *F. oxysporum* Fo47b10.

None of the bacterial strains inoculated simultaneously with the pathogen reduced disease severity on its own (Fo47b10/WCS816 inoculum ratio, 0:1) or in association with nonpathogenic *F. oxysporum* Fo47b10 introduced in the rockwool at a ratio of 1:1 (Fig. 2). The biocontrol with higher densities of Fo47b10 (ratios of 10:1 and 100:1) was significantly improved by adding bacterial strain WCS358. At the highest inoculum ratio of Fo47b10 to WCS816 (100:1), the percentage of diseased plants was reduced 61.1% with Fo47b10 alone down to 28.3% with Fo47b10 plus WCS358. The addition of the Sid− mutant JM218 to nonpathogenic *F. oxysporum* Fo47b10 did not affect the reduction of disease severity.

**Population densities of antagonistic microorganisms.** For Fo47b10/WCS816 inoculum ratios of 1:1, 10:1, and 100:1, the densities of Fo47b10 measured 3 weeks after its inoculation were 2.5 × 10⁶, 4.3 × 10⁵, 2.2 × 10⁶ CFU ml of rockwool⁻¹, respectively. All of these fungal densities were significantly different (P = 0.05).

The effect of the loss of pseudobactin 358 production on the survival of pseudomonads was assessed by comparing the densities of the wild type (WCS358) with those of the Sid− mutant (JM218) at an Fo47b10/WCS816 inoculum ratio of 10:1. Comparisons of bacterial densities were performed in the absence and presence of nonpathogenic *F. oxysporum* Fo47b10. The densities of Sid− mutant JM218 (4.4 and 4.4 log CFU g of root⁻¹ in the absence and presence of Fo47b10, respectively) did not differ significantly (P = 0.05) from those of the parental strain (4.3 and 4.5 log CFU g of root⁻¹, respectively). The presence of Fo47b10 did not modify the density of wild-type WCS358 or transposon mutant JM218. The density of the Sid− mutant JM218 scored on King’s B agar medium supplemented with kanamycin was not significantly different from the density estimated with a serological procedure (data not shown).

**DISCUSSION**

The results of this study show that fusarium wilt was strongly suppressed by nonpathogenic *F. oxysporum* Fo47b10 combined with *P. putida* WCS358. The suppression is more efficient than that achieved by either antagonistic microorganism alone. Increased suppression with other bacterial strains and other host plants has been described by Lemanceau (18), Lemanceau and Alabouvette (19), and Park et al. (28). The present study demonstrates for the first time the implication of a bacterial metabolite in the beneficial effect of microbial coinoculation.

Previous studies suggested that pseudobactin alone may be responsible for the reduction of fusarium wilt severity. Kloeper et al. (16) established that introduction of partially purified pseudobactin in conducive soil lowers the percentage of wilted plants. Scher and Baker (32) demonstrated that the natural suppressiveness to fusarium wilt of the soil from Salinas Valley is caused by iron competition. Scher and Baker also showed that fluorescent *Pseudomonas* spp. contribute to soil suppressiveness and hypothesized that siderophore production by fluorescent *Pseudomonas* spp. is responsible for the natural suppressiveness of the Salinas Valley soil. Van Peer et al. (37), by manipulating iron availability in soilless culture of carnation, also obtained evidence that siderophore-mediated iron competition can be involved in suppression of fusarium wilt by fluorescent *Pseudomonas* spp. The use of transposon mutants is a
powerful tool for studying the involvement of bacterial metabolites in the biocontrol of soil-borne diseases (5, 6, 11). The possible implication of pseudobactin 358 production in fusarium wilt suppression achieved by nonpathogenic *F. oxysporum* Fo47b10 combined with *P. putida* WCS358 was evaluated in the present study by the use of a siderophore-negative transposon mutant, JM218, of *P. putida* WCS358. The Tn5 mutation resulting in suppression of siderophore production was assumed to be stable because JM218, reisolated from the carnation roots, was resistant to kanamycin and did not synthesize pseudobactin 358. By comparing the growth of plants treated with wild-type WCS358, Tn5 Sid− mutants, or an Sid+ Tn5 derivative, it was previously shown that Tn5 had no detectable effect on iron competition in plants (5). The Sid− mutant JM218 did not improve the biological control achieved by nonpathogenic *F. oxysporum* Fo47b10 at all, whereas the wild type did. The lack of efficacy of the Sid− mutant was not related to reduced root colonization. As suggested by Bakker et al. (7), the absence of reduction in root colonization by the Sid− mutant may be caused by the use of ferric siderophores produced by other pseudomonads. All of these data suggest strongly that the improved biocontrol by nonpathogenic *F. oxysporum* Fo47b10 in the presence of *P. putida* WCS358 is related to the production of pseudobactin 358.

*P. putida* WCS358 did not reduce disease severity when inoculated simultaneously with the pathogen in the absence of nonpathogenic *F. oxysporum* Fo47b10. Lemanceau and Alabouvette (19) observed that some bacterial strains on their own were not effective but did improve the control achieved with nonpathogenic *F. oxysporum*. A previous study of Duijff et al. (12) showed that *P. putida* WCS358 suppressed fusarium wilt of carnations when applied to a natural soil but not when applied to a steamed soil. This difference in efficacy could be related to the soil microflora, which includes nonpathogenic *Fusarium* spp. According to Louvet et al. (24), these fungi are usually present in soils at densities ranging from $10^7$ to $10^8$ CFU g of soil$^{-1}$. Therefore, the absence of any effect of WCS358 alone in the present study may be explained by the very low density of indigenous microflora in rockwool at the time of infestation with pathogenic *F. oxysporum* (36).

For a given density of the pathogen, the efficacy of control of nonpathogenic *F. oxysporum* Fo47b10 was related to its inoculum density. As the ratio nonpathogenic *F. oxysporum* to pathogenic *F. oxysporum* was increased, the disease was more suppressed. However, the suppression seemed to reach a plateau at an inoculum ratio of 10:1. This behavior would suggest that competition between nonpathogenic *F. oxysporum* and pathogenic *F. oxysporum* is implicated in the control achieved by Fo47b10, as proposed by Couteaudier and Alabouvette (37).

Since good survival of nonpathogenic *F. oxysporum* Fo47b10 in rockwool was shown previously (13), its density was only checked 3 weeks after inoculation. The lack of modification of the densities of inoculated bacteria by nonpathogenic *F. oxysporum* Fo47b10 is in agreement with previous results (13). Nevertheless, as suggested by Park et al. (28), introduction of nonpathogenic *F. oxysporum* could still enhance the activity of the inoculated *Pseudomonas* spp. in the rhizosphere.

The antagonistic activity of pseudobactin against fungi has been related to reduction of iron (Fe$^{3+}$) availability (20, 22, 26). According to the present results, the suppression of fusarium wilt by iron competition due to pseudobactin production in cooperation with nonpathogenic *F. oxysporum* is significant.

**ACKNOWLEDGMENTS**

This work was supported in part by a fellowship to P.L. from the North Atlantic Treaty Organization and in part by Netherlands Technology Foundation project UBI 38.0390.

**REFERENCES**

17. Leeman, M., J. M. Raaijmakers, P. A. H. M. Bakker, and B.


